Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar
http://dx.doi.org/10.5424/sjar/2012104-2754

Spanish Journal of Agricultural Research 2012 10(4), 1117-1120
ISSN: 1695-971-X
eISSN: 2171-9292

Short communication. *Rhizoctonia solani* as causal agent of damping off of Swiss chard in Spain

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Abstract

During September 2011, post-emergence damping off of Swiss chard (*Beta vulgaris* subsp. *cicla* L.) was observed in a greenhouse in Villa del Prado (Spain). About 20% of the seedlings showed damping off symptoms. Lesions were initially water soaked, dark brown necrosis of crown tissue, irregular in shape and sunken in appearance on large plants, causing the infected seedlings to collapse and eventually die. *Rhizoctonia solani* was isolated consistently from symptomatic plants. After morphological and molecular identification of the isolates, pathogenicity was tested by placing agar plugs of four isolates adjacent to the stem at the three or four true leaf stage. In inoculated plants, brown crown and stem necrosis occurred while control plants did not show disease symptoms. Pathogenicity using non-germinated seeds was also tested. All four isolates produced extensive damping off when inoculated on non-germinated seeds. To our knowledge, this is the first report of damping off of Swiss chard caused by *R. solani* in Europe.

Additional key words: *Beta vulgaris* subsp. *cicla*; damping-off; morphological identification; molecular identification; silver beet.

Resumen

Comunicación corta. *Rhizoctonia solani,* agente causal de la muerte de plántulas de acelga en España

En el mes de septiembre de 2011, se observó muerte de plántulas en acelgas cultivadas en un invernadero en Villa del Prado (España). Cerca del 20% de los semilleros mostraban síntomas de caída de plántulas. Las lesiones iniciales tenían un aspecto húmedo y consistían en una necrosis del tejido de la corona de color marrón oscuro, de bordes irregulares y deprimidos en las plantas adultas, produciendo el colapso de las plántulas y, ocasionalmente, la muerte de las mismas. *Rhizoctonia solani* se aisló de manera consistente de las plantas sintomáticas. Tras una identificación morfológica y molecular, la patogenicidad de los aislados se probó colocando discos de agar adyacentes al tallo de la planta en estados de tres o cuatro hojas verdaderas. En las plantas inoculadas se observó un oscurecimiento de la corona y la necrosis del tallo en todos los casos, síntomas que no mostraron las plantas control. Por otro lado, se comprobó la patogenicidad de los aislados en semillas no germinadas. Los cuatro aislados produjeron muerte de plántulas cuando se inocularon en semillas no germinadas. Hasta donde hemos podido comprobar, este trabajo parece ser la primera cita de muerte de plántulas de acelga causada por *R. solani* en Europa.

Palabras clave adicionales: acelga; *Beta vulgaris* subsp. *cicla*; caída de plántulas; identificación molecular; identificación morfológica.

Beta vulgaris subsp. *cicla* L. is an annual crop widely grown in the Mediterranean region. It is a tall leafy green vegetable commonly referred to as Swiss chard which belongs to the same botanical family as beets and spinach and have an exceptionally number of health-promoting nutrients. This vegetable may be seeded directly in the soil or transplanted from a seedbed which offers an interesting enlargement of the current vegeta-

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Received: 10-02-12. Accepted: 29-10-12

Abbreviations used: DSI (disease severity index); PCR (polymerase chain reaction); PDA (potato dextrose agar).

ble assortment by growing as a vegetable for its edible leaves and stalks. Swiss chard is also a very nutritive demanding species (Pokluda & Kuben, 2002).

Swiss chard area in Europe is reduced. Annual Spanish Swiss chard production is around 66,500 tons grown on 2,100 ha. Villa del Prado is the main production area within the Community of Madrid with 40.5 ha and a total annual production of 9,624 tons. In Villa del Prado (Madrid), chard harvest is manual and staggered, removing the outer leaves, allowing the inner ones to grow, so most tender leaves are harvested (Hoyos *et al.*, 2005).

In September 2011, symptoms of damping-off were observed on approximately 20% of the plants at the stem base around the soil line of Swiss chard seedlings of cv. Lyon Yellow in a greenhouse (800 m^2, tunnel type) in Villa del Prado (Spain) where seedlings were transplanted. The symptoms were not detected in any other greenhouses in the area. Lesions were initially water soaked, dark brown necrosis of crown tissue, irregular in shape, and sunken in appearance on large plants, causing the infected seedlings to collapse and eventually die. These symptoms were similar to those caused by *R. solani* in other crops and prompted us to determine the etiology of this damping-off. *R. solani* has been previously reported to cause damping-off of *Beta vulgaris* subsp. *cicla* L. in California (USA) (Koike & Subbarao, 1999) and China (Yang *et al.*, 2007), and it has been previously reported in Spain in many crops, for example in strawberry nurseries (Duhart *et al.*, 2000; De Cal *et al.*, 2004), cotton (Melero-Vara & Jimenez-Díaz, 1990), potato (Sardiña, 1945) and French beans (Tello *et al.*, 1985; Sinobas *et al.*, 1994).

Small pieces of symptomatic lower stem and roots of 25 symptomatic plants were surface disinfected in sodium hypochlorite (0.5% w/v) for 2 min and air dried. The sections were then placed on PDA (potato dextrose agar) medium and a selective media for Oomycetes (Ponchet *et al.*, 1972) and incubated for 5 days at 25°C.

Isolations from diseased stem and root tissue consistently yielded fungal colonies light gray to brown with abundant growth of mycelia and dark brown sclerotia in 92 and 84% of the samples plated on PDA and Ponchet media, respectively. The hyphae tended to branch at right angles when examined under microscope. A septum was always present in the branch of hyphae near the original point and slight constriction at the branch was observed. Isolates were morphologically identified as *Rhizoctonia solani* (Sneh *et al.*, 1991). *Pythium* spp. and *Ulocladium* spp. were also isolated from the samples but always in a very low percentage of samples (< 8%).

Molecular identification was performed by sequencing the region ITS1-5.8S-ITS2 of the rDNA. PCR amplifications were carried out using the primer set ITS1/ITS4 and the conditions described by White *et al.* (1990). The fragments obtained were subsequently sequenced in both directions.

Subsequent database searches by the BLASTN software indicated that the resulting sequence of 526 bp had a 100% identity with the corresponding gene sequence of *R. solani* anastomosis group (AG) 4, a common soil fungus with a wide host range that causes a number of plant diseases. The sequences were deposited on the EMBL Sequence Database (Accession numbers HE655451, HE655450, HE655449).

Four *R. solani* isolates, the main pathogen isolated from diseased plants were tested in pathogenicity assays. These isolates were maintained on PDA media and stored at 4°C in the fungus collection of the Plant Production Department of the Technical University of Madrid. Pathogenicity was confirmed through inoculation of healthy Swiss chard plants cv. Lyon Yellow, commonly used in the area. Four-week-old plants were grown on 1000 mL plastic greenhouse pots, previously filled with a disinfected (twice autoclaved 105 kPa, 30 min at 120°C) mix of vermiculite and peat (1:1). Plants were inoculated with each of the isolates by placing a 5-mm PDA plug of mycelia at the stem base and covering with a thin layer of substrate. Another four plants treated with non-inoculated PDA served as control. The experiment was repeated. After inoculation, the plants were maintained at 23-28°C and a 14 h photoperiod of 10,000 Lux cool white fluorescent light. Disease symptoms were graded into five classes as follows: 0 = no symptoms; 1 = reddish brown colored crown; 2 = necrosis of crown tissue only; 3 = necrosis of crown and the leafstalks; 4 = crown completely rooted with defoliation; 5 = completely rotted dead plant. A disease severity index (DSI) was calculated as the mean of four plants for each isolate and test replicate. Disease symptoms were recorded 3 weeks after inoculation. At the end of the experiment, plants were oven-drying at 80°C for 48 h before dry weight was determined.

The same four selected *R. solani* isolates were used to test their effect on Swiss chard seeds germination and occurrence of damping off disease of cv. Lyon Yellow.

Seeds were surface disinfested in NaOCl (40-50 mg L^{-1} active Cl_2) for 3 min, rinsed five times in sterile distilled water and placed on surface disinfested 36 × 52 × 7 cm plastic trays previously filled with two-thirds capacity of an autoclaved (105 kPa, 30 min at 120°C) vermiculite substrate (Agroalse S.L., Moncada, Valencia, Spain). Mycelium from one actively growing 10-14 days old culture on PDA incubated at the same conditions indicated above was homogenized in sterile distilled water and a volume of 200 mL was added to each tray seeded with 50 non-germinated seeds. Following inoculation, seeds were covered with a 1 cm layer of autoclaved vermiculite. Control seeds were treated with PDA homogenized in sterile water. Inoculated and control plants were maintained at 20-25°C and a 14 h photoperiod of 18.8 µE m^{-2} s^{-1}. After 14 days seedlings were rated for damping off as described by Schumann & D'Arcy (2006) following the recommendations of the International Seed Testing Association standards (ISTA, 2004). The experiment was repeated.

Data collected in experiments were subjected to one way ANOVA, with DSI or weight as dependent variable, and isolate as independent variable. Parametric analyses (one-way ANOVA) were used when Levene's test indicated no significant heterogeneity of variance. Non-parametric analyses (Kruskal-Wallis test) were used when control treatment was not included in the analyses and the heterogeneity of variance was significant. Similar analyses were carried out for seed germination. All calculations were carried out using StatsGraphics Centurion XV.II (Statistical 195 Graphics Corp., Herndon, VA, USA).

Symptoms of inoculated plants included wilting and brown to black necrosis of the lower taproot of three or four leaf stage chard. Water-soaked, brown lesions, identical to the symptoms described above, were observed on the stem base of all inoculated plants, whereas no symptoms developed on the control plants. The fungus was isolated from affected crown samples, and the identity was confirmed by microscopic appearance of the hyphae, fulfilling Koch's postulates. This pathogenicity test was conducted twice.

DSI values from inoculated Swiss chard were always higher than 2.5 and they were not significantly different (p = 0.374) among isolates (Fig. 1A). All *R. solani* inoculated isolates caused dry weight reductions on inoculated chard plants when compared to that estimated for control plants (Fig. 1B). Isolate 1 caused the most severe decreases, with up to a 44% significant reduction ($p < 0.05$) in dry weights of seedlings, but isolate 2 did not cause significant reductions as compared to control plants.

The same isolates were tested in pathogenicity test in un-germinated seeds. All four isolates produced extensive damping-off on pre-germinated seeds without significant differences among them (Fig. 2), with a disease incidence above 90% for the different pathogen isolates.

Successful control of *Rhizoctonia* damping-off remains a serious problem for cucumber and melon cultivation in Spain (Tello *et al.*, 1990) with the pathogen being reported in different crops in the last few years (El Bakaki, 2000; Delgado *et al.*, 2005). *R. solani* has been previously reported to cause

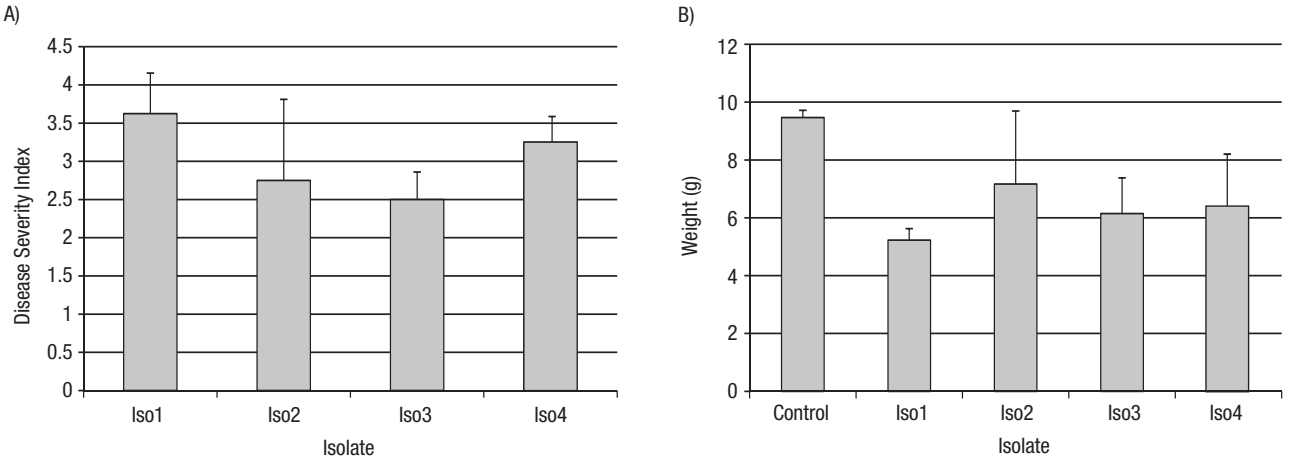

Figure 1. Disease severity index (A) and dry weight (B) of Swiss chard (*Beta vulgaris* ssp. *cicla*) seedlings following artificial inoculation with four isolates of *R. solani*. Thin bars indicate the standard deviation of the data.

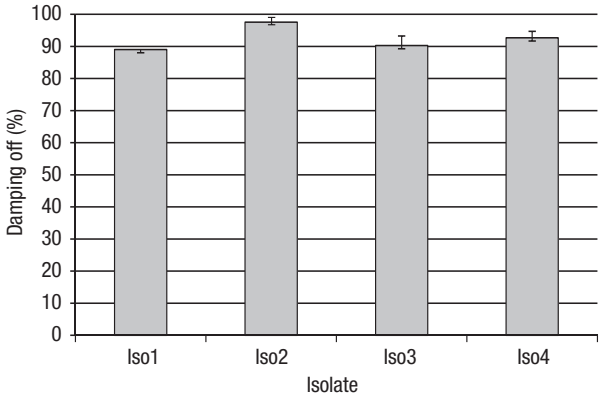

Figure 2. Effects of inoculation with four isolates of *Rhizoctonia solani* on damping off in Swiss chard (*Beta vulgaris* ssp. *cicla*). Thin bars indicate the standard deviation of the data.

damping-off of *B. vulgaris* subsp. *cicla* L. in California, USA (Koike & Subbarao, 1999) and China (Yang *et al.*, 2007).

The current losses of *R. solani* have not been accurately evaluated in the production area of Swiss chard in Spain but taken into account our results it seems necessary to be done in the near future. To our knowledge, this is the first report of damping-off caused by *R. solani* on Swiss chard in Europe. With the extended use of Swiss chard in crop rotations with some vegetables such as cucumber, the occurrence of the damping-off pathogen needs to be taken into account when designing disease management programs or when selecting crops for rotations.

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